HEPES 10 (pH 7.2; 285–295 mOsm). At the end of the recording, the cell's content was aspirated under visual control into the recording pipette and expelled into a test tube where reverse transcription was performed in a final volume of 10 µl (ref. 16). We then performed two steps of multiplex PCR. The complementary DNAs present in 10 µl of the reverse transcription reaction and corresponding to GAD65, GAD67, ChAT and enkephalin were first amplified simultaneously using the primer pairs previously described[15,17,27], Taq polymerase (2.5 U, Perkin Elmer-Cetus) and 10 pmol of each of the primers were added to the buffer supplied by the manufacturer (final volume, 100 µl) and 20 cycles (94 °C, 30 s; 60 °C, 30 s; 72 °C, 35 s) of PCR were run. We then carried out second rounds of PCR using 2 µl of the first PCR product as template. In this second round, each cDNA was individually amplified using its specific primer pair by performing 35 PCR cycles. We then ran 10 µl of each individual PCR reaction product on a 1.5% agarose gel using ϕx174 digested by *Hae*III as markers for relative molecular mass and stained with ethidium bromide. Genomic DNA amplifications, which can occur when the nucleus is harvested, can be easily differentiated from cDNA amplifications by a size criterion. Indeed, for each primer pair, the sense and antisense primers are positioned on two different exons.

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Correspondence and requests for materials should be addressed to M.M. (e-mail: michel.muhlethaler@medecine.unige.ch).

TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease

Jane A. Gross*, Janet Johnston*, Sherri Mudri*, Rachel Enselman*, Stacey R. Dillon*, Karen Madden†, Wenfeng Xu†, Julia Parrish-Novak‡, Don Foster†, Cathy Lofton-Day§, Margaret Moore§, Alisa Littau‡, Angelika Grossman‡, Harald Haugen‡, Kevin Foley§, Hal Blumberg§, Kim Harrison*, Wayne Kindsvogel* & Christopher R. Clegg*

*Departments of *Immunology, † Functional Cloning, ‡ in Vivo Biology and § Genetics, ZymoGenetics, 1201 Eastlake Avenue East, Seattle, Washington 98102, USA*

B cells are important in the development of autoimmune disorders by mechanisms involving disregulated polyclonal B-cell activation, production of pathogenic antibodies, and co-stimulation of autoreactive T cells. zTNF4 (BLyS, BAFF, TALL-1, THANK)[1–5] is a member of the tumour necrosis factor (TNF) ligand family that is a potent co-activator of B cells *in vitro* and *in vivo*[1,2,5]. Here we identify two receptors for zTNF4 and demonstrate a relationship between zTNF4 and autoimmune disease. Transgenic animals overexpressing zTNF4 in lymphoid cells develop symptoms characteristic of systemic lupus erythaematosus (SLE) and expand a rare population of splenic B-1a lymphocytes. In addition, circulating zTNF4 is more abundant in NZBWF1 and MRL-*lpr/lpr* mice during the onset and progression of SLE. We have identified two TNF receptor family members, TACI[6] and BCMA[7,8], that bind zTNF4. Treatment of NZBWF1 mice with soluble TACI–Ig fusion protein inhibits the development of proteinuria and prolongs survival of the animals. These findings demonstrate the involvement of zTNF4 and its receptors in the development of SLE and identify TACI–Ig as a promising treatment of autoimmune disease in humans.

zTNF4 was identified in a human-granulocyte-derived complementary DNA library by homology with other known TNF ligands[9]. Soluble recombinant zTNF4 stimulates proliferation of human B cells in synergy with other B-cell activators, augments immunoglobulin production, and upregulates expression of cell-surface molecules involved in B-cell effector function (refs 1, 2 and data not shown). Recent studies demonstrate that expression of BAFF, in the liver of transgenic mice, results in lymphoid disorders and autoimmune manesfestations[5]. We obtained similar results expressing zTNF4 in transgenic mice using a lymphoid specific V_H promoter[10] and Eμ enhancer[11] (zTNF4-TG). We identified expression of the transgene in 15 founder animals by polymerase chain reaction with reverse transcription (RT–PCR) and a zTNF4 enzyme-linked immunosorbent assay (ELISA) detected increased levels of circulating zTNF4 protein in these animals (data not shown). Flow cytometric analysis revealed a marked increase in the proportion and total number of $B220^+$ B cells in the spleen and lymph node relative to controls (Fig. 1a) and an increased percentage of syndecan$^+$ plasma cells (Fig. 1b). However, there was no apparent effect of zTNF4 overexpression on development of $B220^+$ IgM^- progenitor B cells in bone marrow (data not shown). The total number of splenic T cells in the transgenic animals was normal, but the $CD4^+$ and $CD8^+$ T-cell populations displayed an activated phenotype defined by decreased levels of LECAM-1 and increased CD44 (data not shown).

We analysed the levels of immunoglobulin collected from 15 founders and 9 offspring ranging from 6 to 23 weeks of age, and determined that the amount of both IgM and IgG was elevated at least threefold in over 50% of the animals tested (IgM increased

more than IgG). IgE levels were also increased in 18% of the serum samples from the zTNF4-TG mice (Fig. 1c). Significantly, several older zTNF4-TG animals developed high titres (1:1,600) of anti-double-stranded (ds) DNA antibodies (Fig. 1c), proteinuria (data not shown) and glomerulonephritis (Fig. 1d), suggesting that unregulated expression of zTNF4 can contribute to the development of SLE-like symptoms.

Thus, the phenotype of zTNF4-TG mice is similar to that of BAFF transgenic mice[5]. Unlike BAFF transgenic mice, zTNF4-TG mice also contained elevated numbers of splenic B-1a cells ($CD5^+$, $B220^{lo}$ and IgD^{lo}) (Fig. 1b). This B-cell lineage is thought to produce predominantly self-reactive IgM antibodies and represents only a small fraction of splenocytes (0.5%) in normal mice[12]. We also identified a population of $CD19^+$ B cells (10–15%) in thymuses from 3 out of 15 zTNF4 founders (Fig. 1b) that may represent expansion of resident B cells or recirculating peripheral B cells. B cells were found to accumulate in other tissues including the lung and small intestine (data not shown).

To characterize the association of zTNF4 with SLE further, we measured zTNF4 protein levels in serum from NZBWF1 and MRL-*lpr/lpr* mice. Both mouse strains develop chronic, spontaneous autoimmune disease[13]. As a baseline, serum zTNF4 was measured in female NZBWF1 animals before onset of disease (10 weeks of age) and then measured during disease progression (Fig. 2a). An average threefold increase in serum zTNF4 was detected in 20% of the animals at 14 weeks of age, and reached sixfold over baseline in 100% of the animals by 24 weeks of age. zTNF4 levels were highest (ninefold over baseline) in animals with

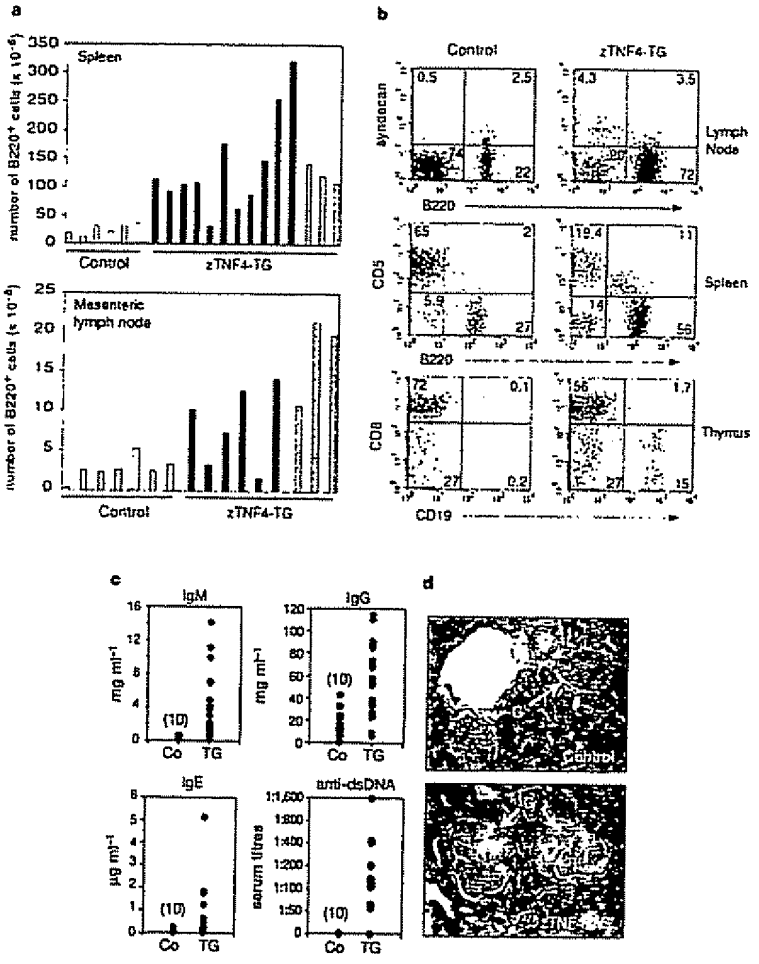

Figure 1 zTNF4 transgenic animals develop a phenotype characteristic of SLE. a, The total number of B220⁺ lymphocytes was determined by fluorescence-activated cell sorting (FACS) in cell suspensions prepared from spleen and mesenteric lymph nodes. Each bar represents data from individual control mice (open bars), 13-week-old zTNF4 transgenic (zTNF4-TG) founder animals (shaded bars) and 11-week-old transgenic offspring (hatched bars). b, Cells were isolated from lymph node (top), spleen (middle), and thymus (bottom) from zTNF4-TG or control mice and analysed by FACS with the indicated antibodies. Data are representative of 11 individual zTNF4-TG founders, 3 offspring and 6 control mice analysed. c, IgM, IgG and IgE and anti-dsDNA titres were assayed by ELISA in 23 serum samples from 14 zTNF4-TG founder animals ranging from 6 to 23 weeks of age, and from 12 age-matched littermate control animals (Co). d, Kidney sections from 15-week-old control (top) or zTNF4-TG (bottom) were stained with haematoxylin and eosin. Amyloid deposition and thickened mesangium of the glomeruli (G) were identified. A representative of 4 zTNF4-TG founders and of 12 control mice is shown.

markedly increased titres of anti-dsDNA antibodies and elevated proteinuria (≥ 2,000 mg dl^{-1}). In contrast, serum zTNF4 was not elevated in the healthy NZB/B parental strain, even at 30 weeks of age. MRL-*lpr/lpr* mice are a more severe model of SLE than the NZBWF1 strain, and rapidly develop autoimmunity and profound lymphoproliferative disease[14]. Serum zTNF4 was increased in diseased MRL-*lpr/lpr* mice compared with the amount detected in healthy 11-week-old MRL/MpJ mice (Fig. 2b). However, the relative increase in zTNF4 was similar in both NZBWF1 and MRL-*lpr/lpr* strains when comparing healthy controls with diseased animals.

The receptor for zTNF4 appears to be expressed predominantly on B cells and on activated T cells[6]. Biotinylated zTNF4 was found to bind to almost all mature CD19$^+$ peripheral B cells, weakly to immature B cells in the bone marrow, and to most transformed B-cell lines, including RPMI 1788, Raji and Ramos (data not shown). A cDNA expression library, prepared from RPMI 1788 messenger RNA, was transfected into COS7 cells and receptor-positive cells were identified with labelled zTNF4. Two orphan TNF receptor family members, TACI[6] and BCMA[7,15], were isolated. TACI is expressed on B cells and signals through CAML, activating the transcription factors NF-AT, AP-1 and NFκB[6]. BCMA is a B-cell maturation antigen that is thought to be an intracellular protein[7,15]. Alignment of TACI and BCMA sequences reveals significant homology between the two receptors within the prototypic cysteine-rich domains[8] (Fig. 3a), but not with other members of the TNF receptor family. zTNF4 binding to other TNF receptor family members was tested using surface-enhanced laser desorption and ionization (SELDI) technology. zTNF4 failed to bind 11 out of the 19 known human TNF receptor family members including CD40, p55 TNFRI, p75 TNFRII, Fas, TRAIL R1, R2, R3, R4, OPG and HVEM (data not shown).

Specific binding experiments of ^{125}I-labelled zTNF4 were performed on baby hamster kidney (BHK) transfectants expressing either TACI or BCMA. Scatchard plot analysis revealed that TACI and BCMA bound labelled ligand with similar dissociation constants (K_d): 1.25 nM for TACI and 1.11 nM for BCMA (Fig. 3b). This result indicates that BCMA can be expressed on the cell surface under these transfection conditions. Soluble versions of TACI and BCMA were made by fusing their extracellular domains to the Fc portion of human IgG1 (TACI–Ig and BCMA–Ig). These fusion

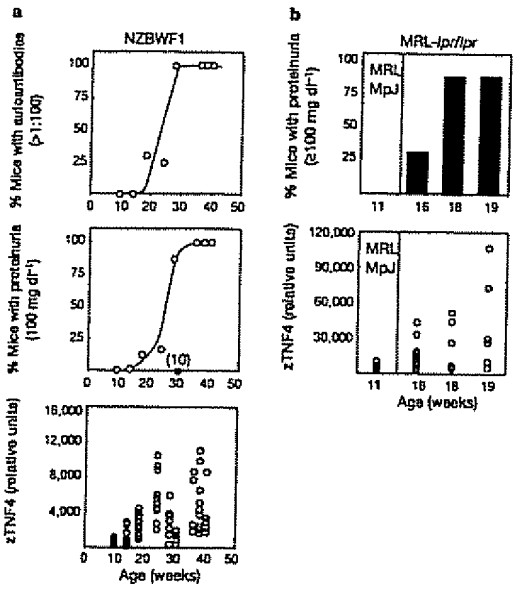

Figure 2 Amount of zTNF4 protein is increased in serum from diseased NZBWF1 mice and MRL-*lpr/lpr* mice. a, Autoantibody titres, proteinuria and zTNF4 levels were measured in 68 NZBWF1 mice ranging from 10 to 40 weeks old (open circles), and ten 30-week-old NZB/B control mice (filled squares). Percentages of animals with anti-dsDNA antibody titre ≥1:100 (top panel) and proteinuria ≥100 mg dl^{-1} (middle panel) are shown (each circle and square represents an average of five to ten mice). Amount of zTNF4 protein was measured in a capture assay and is presented as relative zTNF4 units for each individual NZBWF1 mouse (open circles) and control NZB/B mouse (open squares). b, Proteinuria (upper panel) and amount of zTNF4 (lower panel) were measured in serum from 23 MRL-*lpr/lpr* mice at ages 16, 18 and 19 weeks and from 10 MRL/MpJ control mice at 11 weeks. Approximate amounts of zTNF4 protein in NZBWF1 (0.1–2 ng ml^{-1}) and MRL-*lpr/lpr* (1–10 ng ml^{-1}) serum were determined using a human zTNF4 standard.

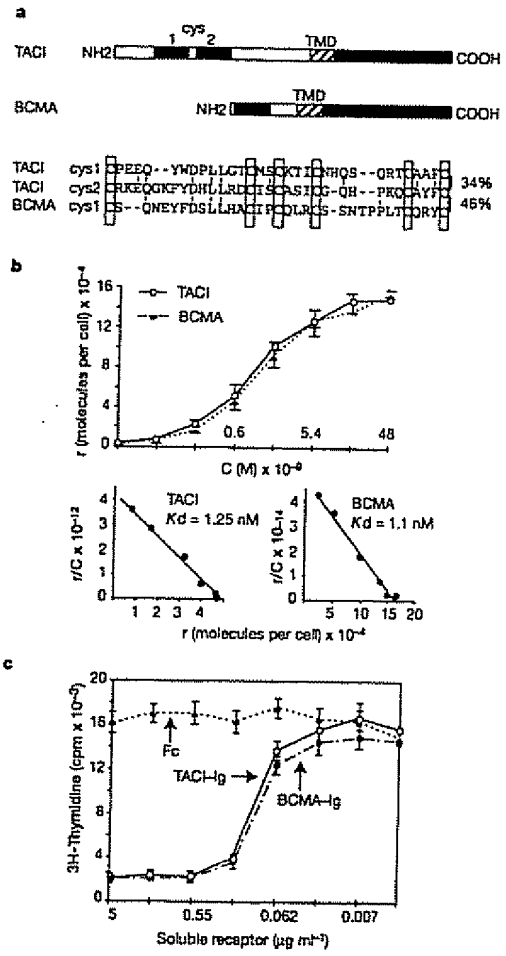

Figure 3 TACI and BCMA are members of the TNF receptor superfamily that bind zTNF4 and can block zTNF4 activity *in vitro*. a, TACI and BCMA. The cysteine-rich repeats (cys, black), transmembrane domain (TMD, hatched) and cytoplasmic domains (shaded) are shown. The cysteine-rich repeats from TACI and BCMA are aligned and the percentage of amino-acid identity is indicated. b, Binding curves and Scatchard plot analysis of ^{125}I-labelled zTNF4 binding to TACI and BCMA were performed using stable BHK transfectants expressing each receptor. c, Human peripheral blood B cells were cultured with zTNF4 (25 ng ml^{-1}), IL-4 (10 ng ml^{-1}) and plate-bound anti-IgM (5 μg ml^{-1}), and treated with increasing amounts of TACI–Ig, BCMA–Ig or control Fc. Cells were cultured for five days and then assayed for cell proliferation by ^{3}H-thymidine incorporation.

proteins prevented zTNF4 binding to human B cells (data not shown) and completely inhibited zTNF4 stimulatory activity on human B cells *in vitro* (Fig 3c) and on murine B cells (data not shown).

To determine the effects of TACI–Ig on the progression of SLE, we treated 21-week-old female NZBWF1 mice (15 mice per group) three times a week for five weeks, with PBS alone, or with 100 μg or 20 μg doses of TACI–Ig or Fc control protein. Treatment with human TACI–Ig (100 μg dose group) delayed the frequency of proteinuria as defined by a marked reduction in the fraction of animals with proteinuria ≥ 100 mg dl^{-1} for up to 10 weeks after the last treatment (P<0.01 by χ^2-test) (Fig. 4a). There was also a noticeable but less prominent effect of TACI–Ig at 20 μg per dose on the development of proteinuria. TACI–Ig treatment (100 μg per dose) increased the survival of animals, with 100% surviving at 38 weeks of age (12 weeks after the last treatment) compared with 47% survival in the equivalent Fc treatment group (Fig. 4b). Examination of anti-dsDNA titres revealed no significant difference in autoantibody levels between the different groups (data not shown). Although elevated serum levels of antibodies to nuclear constituents is a hallmark of SLE[16], there is clear evidence that supports the role of B cells in disease progression in the absence of secreted immunoglobulins[17]. Alternatively, a longer course of treatment with murine TACI–Ig may be needed to suppress autoantibody titres.

Several mechanisms could contribute to the suppression of disease in the TACI–Ig treated animals. To identify potential alterations in the lymphoid compartment of the TACI–Ig treated SLE mice, we determined the percentage of peripheral blood B cells (CD5$^-$, B220$^+$), T cells (CD5$^+$, B220$^-$) and monocytes (CD11b$^+$, B220$^-$) by flow cytometry at 28, 31 and 37 weeks of age (Fig. 4c). There was a significant decrease (P<0.01 by Student's t-test) in the percentage of peripheral blood B cells at 28 weeks of age in animals treated with 100 μg per dose TACI–Ig (decrease of 53%) and 20 μg per dose TACI–Ig (decrease of 32%) compared with percentages of B cells in dose-matched Fc controls. This reduction in B cells persisted five weeks after the last treatment (31 weeks of age) in the high dose TACI–Ig treatment group and returned to levels found in the control groups at 37 weeks of age. These results indicate a role for zTNF4 in maintaining peripheral B-cell populations that have a pathogenic effect on the development of renal disease.

B cells play a significant role in the development of autoimmunity, through B-cell activation, production of pathogenic antibodies and co-stimulation of autoreactive T cells. zTNF4 is a potent molecule, capable of stimulating B cells and driving B-cell differentiation to a pathogenic state. Understanding the regulation of zTNF4 expression and the biology of its receptors, TACI and BCMA, will be important for understanding B-cell development in the normal animal and during disease states. □

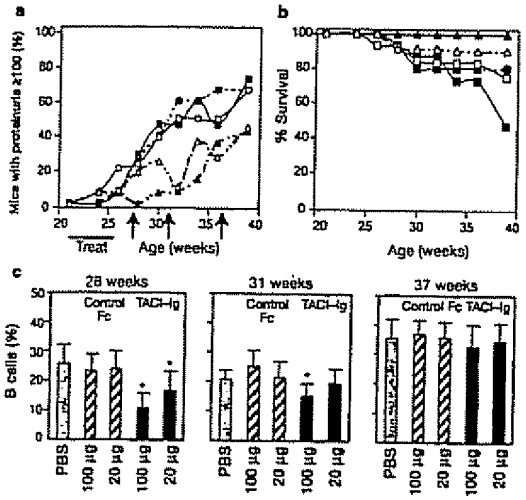

Figure 4 TACI–Ig inhibits the progression of proteinuria and increases the survival of NZBWF1 mice. a, Female NZBWF1 mice (15 mice per group) at 21 weeks of age were injected intraperitoneally three times a week for five weeks with PBS (filled circles), control Fc protein at 100 μg per dose (filled squares) or 20 μg per dose (open squares), or TACI–Ig at 100 μg per dose (filled triangles) or 20 μg per dose (open triangles). Proteinuria was assessed every 2 weeks and the mice were bled at 28, 31 and 37 weeks of age (arrows) to determine the percentage of peripheral blood B cells. The percentage of mice in each group with proteinuria ≥ 100 mg dl^{-1} is shown. b, The percentage of mice surviving in each treatment group is plotted as a function of their age. c, The percentages of viable B220$^+$CD5$^-$ B cells in the peripheral blood of mice in each group were determined by flow cytometry at the time points indicated; standard deviations for each group are shown. Statistically significant differences (Student's t-test: P<0.01) between TACI–Ig treatment and dose-matched Fc control groups are identified (asterisk).

Methods

Recombinant proteins

To produce soluble zTNF4, a DNA fragment containing the sequences for the yeast alpha factor pre-pro leader, a Flag epitope, and the extracellular domain (residue 141–285) of zTNF4 was cloned downstream of the AUG1 promoter into a modified form of the *Pichia methanolica* expression vector pCZR134 (ref. 18). Protein was purified from yeast ferments on an anti-Flag affinity column. DNA encoding the extracellular domain of human TACI (residue 1–154, ref. 6) or BCMA (residue 1–48, ref. 7) was fused to the Fc region of human Ig heavy chain γ1 by PCR and cloned into a mammalian expression vector using the CMV promoter and the TPA pre-propeptide sequence. Proteins were purified by Protein A chromatography from supernatants of transfected CHO DG44 cells adapted into protein-free media (Life Technology Inc.).

Cell culture

zTNF4 stimulations were performed using human B cells purified from donor peripheral blood mononuclear cells by depletion of CD43$^+$ cells using anti-CD43 magnetic beads (Miltenyi). Cells (1×10^5) were cultured in RPMI 1640 medium, 10% FCS and L-glutamine in round bottom 96-well plates (Corning) with zTNF4, anti-IgM (Southern Biotechnology) and IL-4 (Pharmingen) as described in the figure legends. Ramos, Raji and RPMI 1788 were purchased from ATCC.

Transgenic mice

Microinjections were performed by standard methodology on B6C3F2 fertilized ova with a construct containing the IgV_H promoter and Ig $E\mu$ enhancer spliced upstream of the SV40 16S intron followed by the zTNF4 open reading frame and a human growth hormone polyadenylation signal sequence[19,20]. Transgenic animals were backcrossed to C57BL/6 mice (JAX). Transgene expression was measured in spleen and thymus RNA samples prepared using RNeasy mini-kit (Qiagen). Samples of total RNA (25 ng) were analysed in duplicate by quantitative RT–PCR using oligonucleotides against the human growth hormone 3' untranslated region. A Gene Amp 5700 sequence detector (Perkin-Elmer) was used to quantitate the PCR reactions compared with a standard curve containing hGH RNA. Single-cell suspensions from transgenic lymphoid tissues were prepared and analysed on a FACSCalibur flow cytometer (Becton Dickinson) using saturating amounts of the appropriate FITC-, PE- and/or TriColour (TC)-conjugated monoclonal antibodies (Pharmingen).

Animal studies

Female NZBWF1 mice were purchased from JAX. The animals were monitored for development of proteinuria every two weeks and serum was collected monthly to measure anti-dsDNA antibody titres. Morbidity was checked three times a week and daily after proteinuria levels reached 2,000 mg dl^{-1}.

Antibody ELISAs and proteinuria

Murine serum immunoglobulins were quantitated by standard ELISA techniques using capture antibodies goat anti-IgG (Kirkegaard and Perry), goat anti-IgM (Zymed) or goat anti-IgE (Pharmingen). Captured mouse Ig was detected using horseradish peroxidase (HRP)-labelled goat anti-IgG, anti-IgM (Jackson) and anti-IgE (Pharmingen). Anti-dsDNA antibodies were measured[20] using plates coated with poly(dA:dT) (Sigma) detected with HRP-labelled goat anti-mouse IgG Fc (Cappel). The presence of proteins in mouse urine was measured using Uristix (Ames).

zTNF4 detection

zTNF4 was detected in a solution-phase capture assay developed for the Origen 1.5 analyser (IGEN Inc.). Briefly, 1 μg ml^{-1} of a rabbit anti-zTNF4 polyclonal antibody that was affinity purified and biotinylated was incubated for two hours at 20 °C, with undiluted mouse serum samples or a zTNF4 standard curve diluted into normal mouse serum, and 1 μg ml^{-1} of a ruthenylated conjugate of the antibody. Streptavidin labelled beads were added at a final concentration of 0.1 mg ml^{-1}and incubated for 30 min at room temperature. The samples were analysed on the Origen 1.5 analyser and reported as electro-chemiluminescence (ECL) units.

zTNF4 receptor cloning

cDNA was prepared from RPMI 1788 cells (ATCC) and cloned into an expression vector (pZP7). This library of ten million cDNAs was transfected into COS7 cells. After 24 h, the cells were fixed[31] and probed with zTNF4-biotin, followed by a streptavidin–HRP conjugate, and then incubated with an HRP/tyramide substrate to identify receptor-positive cells. Individual positive cells were recovered by micro-dissection. Plasmid DNA was rescued from detergent extracts and used to transform *Escherichia coli*. Individual plasmids were tested by re-transfecting COS7 cells and probing with the zTNF4 probe.

Binding assays

Human zTNF4t protein was iodinated with ^{125}I-Na (Amersham) using Iodo-beads (Pierce) and purified on a Sephadex G25 PD-10 column (Pharmacia). BHK transfectants expressing either TACI or BCMA (2×10^5 cells) were incubated for two hours at 4 °C with dilutions of ^{125}I-labelled TNF4t (specific activity 5.7×10^6 cpm per picomole) in the presence or absence of 20-fold excess unlabelled zTNF4t, washed and measured in a gamma counter.

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Correspondence and requests for materials should be addressed to J.G. (e-mail: grossj@zgi.com).

Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases

Masahiro Kawabuchi*, Yoshinori Satomi†, Toshifumi Takao†, Yasutsugu Shimonishi†, Shigeyuki Nada ‡, Katsuya Nagai*, Alexander Tarakhovsky§‖ & Masato Okada*‖

*Divisions of * Protein Metabolism and † Organic Chemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
‡ Department of Cell Membrane Biology, Division of Biological Science, Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan
§ Laboratory for Lymphocyte Signalling, Institute for Genetics, University of Cologne, Weyertal 121, D-50931 Cologne, Germany
‖ These authors contributed equally to this work.

The Src family of protein tyrosine kinases (Src-PTKs) is important in the regulation of growth and differentiation of eukaryotic cells. The activity of Src-PTKs in cells of different types is negatively controlled by Csk, which specifically phosphorylates a conserved regulatory tyrosine residue at the carboxy-terminal tail of the Src-PTKs[1–3]. Csk is mainly cytoplasmic and Src-PTKs are predominantly membrane-associated. This raises a question about the mechanism of interaction between these enzymes. Here we present Cbp—a transmembrane phosphoprotein that is ubiquitously expressed and binds specifically to the SH2 domain of Csk. Cbp is involved in the membrane localization of Csk and in the Csk-mediated inhibition of c-Src. In the plasma membrane Cbp is exclusively localized in the GM1 ganglioside-enriched detergent-insoluble membrane domain, which is important in receptor-mediated signalling[4–8]. These findings reveal Cbp as a new component of the regulatory mechanism controlling the activity of membrane-associated Src-PTKs.

The ability of Csk to relocate from the cytosol to the plasma membrane[9] and the involvement of the SH2 and/or SH3 domains of Csk in inhibiting of Src-PTKs[10,11] indicate the existence of a plasma-membrane-associated, Csk-binding molecule that mediates the

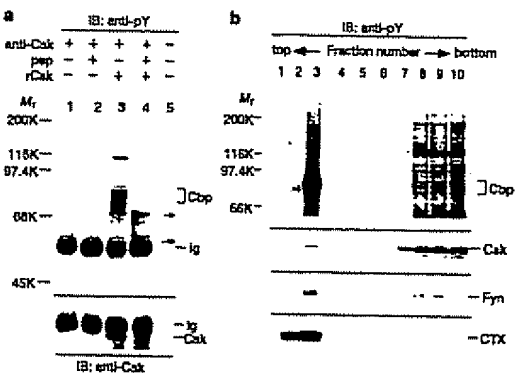

Figure 1 Csk association with the DIM-localized phosphoprotein. a, Association of Csk with M_r 80K–90K phosphoprotein in rat brain lysate. The lysate was incubated with anti-Csk, with or without recombinant Csk (rCsk) and Csk peptide (pep.). The immunoprecipitates (IP) were analysed by immunoblotting (IB) with anti-pY or anti-Csk (bottom). b, Predominant localization of Cbp in the DIM fraction. The Triton X-100 containing brain lysates were fractionated[12]. Aliquots of the fractions were resolved by SDS–PAGE and analysed by immunoblotting with anti-pY, anti-Csk, anti-Fyn and the B-subunit of cholera toxin (CTX).